Uterine immune response after single and double fresh sperm insemination in mares

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Research Article

Uterine Immune Response After Single and Double Fresh Sperm Insemination in Mares [1]

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Abstract

The aim of the study was to evaluate the effects of single and double inseminations on the inflammation of the uterus in mares. Nine mix breed mares with endometrial biopsy scores grade I and IIA, aged 5-15, were used in the study. Two experiments were performed over two estrous cycles: (1) single insemination, (2) double insemination. All mares were used in both insemination group. In the single insemination cycles one insemination was performed 24 h after hCG injection, while in the double insemination cycles two inseminations were carried out 24 h and 48 h after the hCG injection. Endometrial biopsies were collected in both cycles immediately before (=0), and 6, 30 and 54 h after the first insemination. Gene expressions of the inflammatory markers IL-1β, IL-6, IL-8, iNOS, SAA, COX-2 and CASP-3 and histopathological alterations of the endometrium were determined. The 6 h expression levels of IL-6 were higher when compared with 30 h and 54 h ($P<0.05$). CASP-3 expression levels in single insemination cycles were higher at 0 h and 6 h when compared with 54 h ($P<0.05$). In conclusion expression levels of the IL-1β,-6,-8, iNOS, SAA and COX-2 were not different between single or double insemination cycles. This study provides preliminary evidence to further characterize the changes in the expression of relevant genes in response to fresh semen.

Keywords: *Mare, Postbreeding endometritis, Proinflammatory cytokines, Insemination*

Kısraklarda Tek ve Çift Taze Sperm İle Tohumlama Sonrası Uterin İmmun Yanıt

Özet

Çalışmada kısraklarda tek ve çift tohumlama sonrası immun yanıt değerlendirildi. Farklı ırktan, 5-15 yaşlarında ve endometriyal biyopsi skoru I ve IIA olan 9 adet kısrak kullanıldı. İki östrus siklusunda iki uygulama yapıldı: (1) tek tohumlama (2) çift tohumlama. Herbir kısrak tek ve çift tohumlama olmak üzere iki deney grubunda da yer aldı. Tekli uygulamalarda hCG uygulaması sonrası 24. saatte tohumlama yapılıyorken; çiftli uygulamalarda hCG uygulaması sonrası 24. ve 48. saatte tohumlama yapıldı. Tohumlamadan hemen önce (0.) ve tohumlamadan sonraki 6., 30. ve 54. saatte uterus biyopsi örnekleri alındı. Yangı parametrelerinden IL-1β, IL-6, IL-8, iNOS, SAA, COX-2 ve CASP-3 mRNA ekspresyonları ve endometriumdaki histopatolojik değişimler değerlendirildi. Tekli gruptaki kısraklarda 6. saatdeki IL-6 mRNA expresyonları 30. ve 54. saatlerdeki değerlere göre yüksek bulundu ($P<0.05$). 0. ve 6. saatlerdeki CASP-3 ekspresyonları ise 54. saatdeki değerlere göre yüksek bulundu ($P<0.05$). Sonuç olarak tekli ve çiftli gruplar arasında IL-1β,-6,-8, iNOS, SAA ve COX-2 expresyonları açısından fark bulunmadı. Taze semen ile yapılan tohumlamalardan sonra bahsi geçen gen expresyonlarına bakarak immun yanıt hakkında ön fikir elde edilebileceği anlaşıldı.

Anahtar sözcükler: *Kısrak, Tohumlama sonrası endometritis, Yangı öncesi sitokinler, Tohumlama*

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INTRODUCTION

Artificial insemination (AI) and natural breeding, may cause local inflammation of the uterus in mares [1]. Postbreeding endometritis is a physiologic event in mares after artificial insemination (AI) or natural mating and serves to eliminate excessive sperm and bacteria introduced into the uterus during breeding. Breeding fertile mares results in endometritis with a transient neutrophilic inflammation that typically is resolved within 48 hours following the breeding [2].

Shortly after breeding or introduction of pathogens to the uterine lumen, polymorphonucleated neutrophils (PMN) migrate into the uterine lumen and intrauterine fluid containing inflammatory mediators accumulates [3]. Activation of the innate immune system acts in combination with mechanical clearance to assist the uterine lumen to clear the local inflammatory response following breeding. Mares differ from each other in terms of their ability to eliminate inflammation and related harmful inflammatory products [4]. Although the causes for these differences in the ability to clear inflammation are unknown, a defective or poorly orchestrated innate immune response has been suggested [5].

The characteristics and grade of endometrial inflammation may vary depending on the sperm number and concentration, seminal plasma, semen extender and number of insemination [6,7]. If mares are inseminated twice with an interval of 24 h, sperm in the second inseminate induce directly an inflammatory response. Inflammatory environment impairs sperm motility and sperm binding to PMN, which results in formation of slow-moving big clusters. This causes the number of sperm transported to the oviduct to be reduced in the second insemination [7,8].

Cytokines are intercellular signaling proteins released by both immune and non-immune that play an important role in modulating local and systemic inflammatory responses, and the resolution of the inflammation. IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α), also known as proinflammatory cytokines, modulate the acute phase response that involves potent systemic and local effects [9]. Infiltration of leukocytes and increased synthesis of cytokines in response to insemination are necessary to enhance reproductive success, however, prolonged inflammation after insemination may yield undesired outcomes [5]. Leukocyte infiltration and increased synthesis of cytokines in response to insemination is necessary to enhance the reproductive success [10], but a prolonged inflammation after breeding is considered to have an adverse consequence [1,4].

Serum amyloid A (SAA) is a rapidly responding acute phase protein and a very sensitive marker of inflammation. It is suggested that the expression of SAA in the epithelial surface of organs associated with external environment may play a role as a first line of defense against invasion by microorganisms and injuries [5].

Nitric oxide (NO) is a main mediator of smooth muscle relaxation in different organs including the uterus. The inducible NOS (iNOS) is typically expressed at sites of inflammation, and produces large amounts of NO for a prolonged time. Nitric oxide (NO) may play a role in determining susceptibility of mares to PBIE through an inhibitory effect on uterine contractility [11]. Cyclooxygenase (COX) is the enzyme required for the biosynthesis of prostaglandins from arachidonic acid [12] and it may produce prostaglandin during inflammation [13]. Furthermore, COX-2 is required for cell growth. Cyclooxygenase 2 gene expression is induced by stimuli or mediators [12]. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins [14].

Although the endometrial immune response has been investigated in mares after insemination, there are no reports relating the cytokine in mares after single and double insemination. The aim of the study was to investigate the inflammatory response in mares after single or double insemination by evaluating uterine mRNA expressions of IL-1β,-6,-8, iNOS, SAA, COX-2, CASP-3 and histopathological findings. Based on previous data, it was hypothesized that the inflammatory changes in response to insemination occur in a time related manner and inflammation-related gene expressions may vary according to the number of insemination.

MATERIAL and METHODS

Animals and Study Design

The animal application procedures were approved by the Local Ethics Committee for Experimental Animals (No: 2015-17). Nine mix breed mares with endometrial biopsy scores (Kenney and Doig [15]) of grades I and IIA, aged 5-15, were used in the study. All mares had normal clinical and gynecological characteristics before entering the study. Two experiments were performed over two estrous cycles: (1) single insemination, (2) double insemination *(Fig. 1)*. All mares were used in both insemination group.

As soon as a follicle was measured to be ≥35 mm, the mares were evaluated for the presence of intrauterine polymorphonuclear neutrophils (PMN) using a cytobrush (Minitube, Tiefenbach, Germany). Mares that had more than three PMN in five fields (400x) were excluded from the study. Human chorionic gonadotropin (hCG,1500 IU, I.V.Chorulon, MSD, Turkey) was administered to mares and insemination was performed 24 h after hCG injection. Before the insemination procedure, ultrasonography was performed and PMN were counted once again. Only mares without ovulation with negative cytology were included in the study and inseminated. Any mares that had a positive cytology and/or ovulation before insemination were

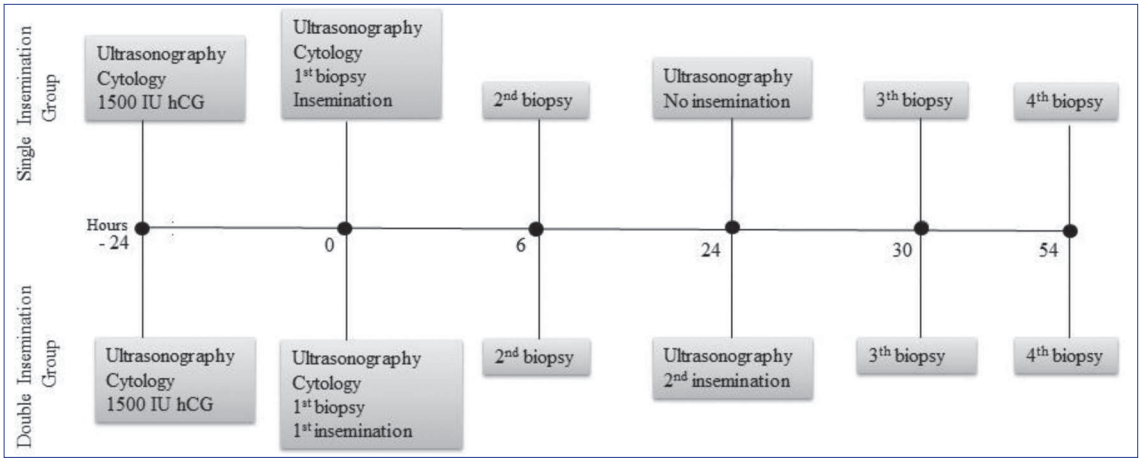

Fig 1. Experimental design of the study

Table 1. *Sequences of the gene-specific primers used in the analysis of gene expression levels*

Target (gene)	Forward Primer Sequence	Reverse Primer Sequence
IL-1β	5′- CGTTTCCCAGAGCCAATCCT -3′	5′- TGCTCATCAGAAGCTGGGTG -3′
IL-6	5′- ATGGCTACTGCTTTCCCCAC -3′	5′- GGCAGAGATTTTGCCGAGGA -3′
IL-8	5′- TGCTTTCTGCAGCTCTGTGT -3′	5′- TGTGGCCCACTCTCAATCAC -3′
iNOS	5′- CCCTTCAACGGCTGGTACAT -3′	5′- CAGCTTGTGTGTTTCCAGGC -3′
SAA	5′- GTTCACAGGCCTCGTCTTCT -3′	5′- TAGCATGTCCCAAGTCCCTC -3′
COX-2	5′- GATCCTAAGCGAGGTCCAGC -3′	5′- AGGCGCAGTTTATGCTGTCT -3′
CASP-3	5′- TCATCCAGTCGCTTTGTGCT -3′	5′- CCATGGCTACCTTGCGGTTA -3′
B2M	5′- CTACTCTCCCTGACTGGCCT -3′	5′- ATTCTCTGCTGGGTGACGTG -3′
GAPDH	5′- CATCAAATGGGGCGATGCTG -3′	5′- ACATTGGGGCATCAGCAGAA -3′
ACTB	5′- GGGCCAGAAGGACTCATACG -3′	5′- TCGATGGGGTACTTGAGGGT -3′

excluded for that cycle, treated, and re-entered the study during a subsequent cycle. In the single insemination group (n=9) mares received one insemination at 0 hour (= 24 h after hCG) while in the double insemination group (n=9) the mares received two inseminations at 0 and 24 h. Ultrasonography was performed again at 24 h and only ovulated mares were included in the study. In this way all mares ovulated between 24-48 h after hCG administration.

Endometrial biopsies were collected at 0, 6, 30 and 54 hours after first insemination. Biopsy samples were obtained at the same time during both cyles. Biopsies were taken from the endometrium using a uterine biopsy forceps (Equivet®, Kruuse, Denmark). Immediately after biopsy, the samples were cut in two pieces with nearly the same size using a sterile scalpel. One portion of the sample was used for histopathological examination and the other portion was used for gene expression analysis. Samples collected for gene expression were snap frozen in liquid nitrogen and kept at -80°C until further processing.

Preparation of Sperm for Insemination

Semen was collected from an Arabian stallion with known fertility. E-Z Mixin BF (ARS, USA) was used as extender. Sperm were counted immediately after collection with Makler Counting Chamber (Sefi-Medical Instruments, USA). The insemination dose contained 500 million progressively motile sperm in 30 mL of extended semen.

RNA Isolation and cDNA Synthesis

RNA isolation was performed by using the RNAzol®RT RNA Isolation Kit (MRC, Canada) according to the manufacturer's recommendations. Briefly, 100 mg of frozen tissue was disrupted and homogenized in 1 mL of RNAzol®RT solution using TissueRuptor (Qiagen, Germany). After the isolation, total RNA was eluted in 50 µL RNase free water. In order to detect the quantity and quality of the total RNA, samples were evaluated using Nano-Drop 2000 (Thermo Scientific, USA). cDNA synthesis was performed using 200 ng of total RNA and SCRIPT cDNA Synthesis Kit (Jena Bioscience, Germany).

Quantitative Real-Time PCR Analysis

Gene-specific primers were designed manually and the sequence homologies were confirmed using the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer sequences are given in *Table 1*.

qRT-PCR was carried out using gene-specific primers, qPCR Green Master with UNG (Jena Bioscience, Germany) and CFX 96 Real Time Instrument (Bio-Rad, Germany) with EVA Green detection module. All samples were studied in duplicate and their average cycle threshold (Ct) values were taken for further analysis. Mean of Ct values of β-actin (ACTB), glydecaldehyde-3-phosphate-dehydrogenase (GAPDH) and β-2-microglobulin (B2M) were used to normalize the values. Analysis of relative expression data was performed by comparative Ct method (2-DDCt method) and the results were expressed as relative quantification (RQ) values. Melting curve analysis was used to confirm the specificity of the amplified products.

For expression analysis in endometrial biopsy samples at different time points and between the two insemination cycles, expression levels of the studied genes (IL-1β,-6,-8, iNOS, SAA, COX-2 and CASP-3) were compared.

Histopathological Examination

Uterine biopsy samples of the mares were fixed in neutral buffered formalin, routinely processed, embedded in paraffin, cut at 4-5 µm thickness and then stained with Hematoxylin and Eosin (H&E) for evaluation by light microscopy.

Biopsy specimens were assessed separately by two pathologists who were blinded to the identification of the samples. Histological inflammatory parameters modified from similar studies such as hyperemia, edema, hemorrhage, surface epithelial disruption (SED), polymorphonuclear leukocyte (PMN) infiltration, lymphoplasmacytic (LP) infiltration and fibrosis and cystic dilation (F+CD) of endometrial glands were evaluated to determine inflammatory status in the uterine tissue. Specimens were scored using a 0-3 scale for each parameter. Score 0 = none; 1 = mild; 2 = moderate; 3 = severe changes [16,17]. While estimating the intensity of PMN, the number of total neutrophilic leukocytes was determined in 3 separate fields at a magnification of 400x. And the scores were given on the basis of the severity of neutrophilia. No neutrophilia (<10 neutrophils in total of three fields; score=0), moderate neutrophilia (10-99 neutrophils in total; score=1), severe neutrophilia (100–149 neutrophils in total; score=2) and very severe neutrophilia (>150 neutrophils in total; score=3) [18].

Statistical Analyses

Repeated measurement of ANOVA in SPSS 10.0 statistical package (SPSS, 1999) was used to analyze data for gene expression. The model included biopsy time as a within-subject effect and insemination as a between-subject effect, and also biopsy time × insemination interaction. Significance control was assessed by using the least significant difference procedure. Comparison between gene expression levels were conducted by using RQ values and results were expressed as mean and standard error (S.E.). Independent samples t-test was applied to compare single versus double uterine insemination at a specific time point for gene expressions.

The Friedman test was used to compare different biopsy time points with respect to different histological parameters in each insemination group and the Wilcoxon test was applied to compare single versus double uterine insemination at a specific time point for histological parameters.

RESULTS

Gene expression of IL-1β,-6,-8, iNOS, SAA, COX-2 and CASP-3 in single and double insemination groups were compared at different time points (0, 6, 30 and 54 h).

Effects of One and Two Inseminations

Expression levels of IL-1β, IL-6, IL-8, SAA, COX-2 and iNOS genes were not different between single or double insemination cycles *(Table 2)*. Additionally, IL-1β, IL-8, SAA and iNOS were similar between different each time point for those gene expressions within the same groups.

IL-6 gene expression in single insemination cycles were higher at 6 h when compared with 30 h and 54 h ($P<0.05$). Although there was a similar trend in double insemination group, it was not significant ($P>0.05$). No differences ($P>0.05$) were detected between single and double insemination group at any time point.

COX-2 mRNA expression in single insemination cycles was increased at 6 h after insemination ($P<0.05$). A significant decrease was obtained at 54 h when compared with 6 h ($P<0.05$). COX-2 mRNA expression in double insemination group did not differ at any time point. No differences ($P>0.05$) were detected between single and double insemination group at any time point.

CASP-3 expression levels in single insemination cycles were higher at 0 h and 6 h when compared with 54 h ($P<0.05$). Double insemination group showed an increased gene expression at 0 h when compared with 30 h ($P<0.05$). Single insemination group had a higher level of expression at 6 h when compared with double insemination group ($P<0.05$).

Comparison of Histopathological Parameters

Hyperemia and edema were observed in all samples immediately after the first insemination application. When the surface epithelial status was assessed, it was detected that the epithelial lining was disrupted *(Fig. 2A)* or infiltrated by PMN at varying levels. PMN was seen right below the surface epithelium, among the uterine glands and in perivascular areas *(Fig. 2B,2C)*. In both insemination groups, PMN increased apparently at the second biopsy time points. Hemorrhage was not a specific finding in either of the samples *(Fig 2D)*.

Table 2. *Gene expressions of inflammatory markers immediately before (hour 0) and 6, 30 and 54 hh after first insemination with fresh sperm during single and double insemination groups. Gene expression values given as mean Relative Quantitation and Standard Error*

Genes	Biopsy Time (hours)	Insemination Procedures		Sig.##
		Single Insemination (n=9)	Double Insemination (n=9)	
IL-1β	0	6.66±2.24	2.24± 0.55	ns
	6	11.29±2.12	6.60±3.12	ns
	30	10.18±3.69	5.98 ±1.42	ns
	54	2.72±0.55	3.21± 1.08	ns
Sig.#		ns	ns	
IL-6	0	5.49±2.37^{ab}	1.22±0.52	ns
	6	8.35±1.95^{a}	4.21±1.42	ns
	30	0.92±0.51^{b}	2.77±1.22	ns
	54	0.71±0.15^{b}	0.77±0.36	ns
Sig.#		*	ns	
IL-8	0	6.95±3.37	1.62±0.83	ns
	6	7.47±2.47	2.72±1.34	ns
	30	5.69±2.81	1.90±0.81	ns
	54	0.81±0.20	0.53±0.26	ns
Sig.#		ns	ns	
CASP-3	0	2.72±0.52^{a}	1.55±0.42^{a}	ns
	6	1.93±0.40^{a}	0.66±0.13^{ab}	*
	30	1.13±0.32^{ab}	0.45±0.86^{b}	ns
	54	0.73±0.12^{b}	0.62±0.10^{ab}	ns
Sig.#		*	*	
SAA	0	33.28±29.05	15.66±7.50	ns
	6	48.97±14.67	154.21±124.26	ns
	30	89.03±64.67	41.50±18.48	ns
	54	81.17±29.69	18.80±11.92	ns
Sig.#		ns	ns	
COX-2	0	1.42±0.46^{a}	1.55±0.53	ns
	6	3.25±0.72^{b}	2.35±1.37	ns
	30	4.25±1.48^{ab}	1.08±0.45	ns
	54	0.75±0.26^{a}	0.85±0.57	ns
Sig.#		*	ns	
iNOS	0	14.31±5.54	30.82±9.09	ns
	6	22.12±7.20	11.00±2.71	ns
	30	25.00±13.78	13.49±3.64	ns
	54	16.59±3.99	25.88±15.27	ns
Sig.#		ns	ns	

Different letters in the same column indicate significant differences. ***Sig.#*** *-values for differences within the same insemination cycle. Sig ## -values for differences between single and double insemination cycles. * P<0.05* ***ns:*** *P>0.05*

Statistically significant differences were noted between single and double insemination at 30th h biopsy with respect to SED (P<0.05), LP infiltration (P<0.05) and F+CD (P<0.05), all of which were found to have increased in the double insemination group.

When biopsy time points in each insemination group were compared among each other, statistically significant differences were detected in terms of edema, SED and PMN. Edema peaked at the 6th h biopsy in the single insemination group and decreased at the 30th h and 54th h biopsy (P<0.05). On the other hand, edema was at the highest level in the first biopsy samples of the double insemination group and decreased at the 6th h biopsy and 30th h biopsy and then increased again at the 54th h biopsy (P<0.05).

SED differed significantly among different time points only in the double insemination group (P<0.05). SED was less severe in the first and last time points when compared with its severity at 6th h biopsy and at 30th h biopsy.

There was a marked significant difference with respect to PMNs both in the single (P<0.05)and double (P<0.001) insemination groups. PMN infiltration was quite low in the first biopsy samples of the single and double insemination groups and increased significantly at 6th h biopsy and at 30th h biopsy. The number of PMN in last biopsy double insemination group was still at a higher level than the first biopsy. All statistical results are given in *Table 3*.

DISCUSSION

Based on this study, we investigated the immune response of endometritis in mares after single or double insemination. The results of the present study demonstrated that mRNA expressions of IL-1β,-6,-8, iNOS, SAA and COX-2 were not different in these two groups of mares.

mRNA expressions of IL-1β, IL-6 and IL-8 tend to be higher at 6 h after insemination in the present study. A gradual increase was found only for IL-6 at 6 h after insemination in the single insemination group. Fumoso et al.[9] reported that mRNA expression of IL-1β, IL-6 and TNF-α for susceptible or resistant showed no variation in 24 h after stimulus of artificial insemination with killed sperm. Resistant mares had lower endometrial mRNA expression for IL-1β and TNF-α in dioestrus than susceptible mares. Twenty-four hours after AI, gene expression for the three cytokines was significantly increased in resistant mares compared to baseline expression in the same mares during estrus of the previous cycle (without treatment). The difference between the results of these two studies might be due to the usage of killed or motile spermatozoa. In additionally, the mares were not classified as susceptible or resistant to persistent breeding induced endometritis (PBIE) but endometrial biopsy scores of all mares were grade I and IIA and they were considered as potential resistant to PBIE.

Gene expressions by equine endometrial cells in response to intrauterine infusion of *E. coli* were investigated in a study. The researchers detected an up regulation of IL-1β, IL-6, IL-8 and TNF-α at 3 h after *E. coli* inoculation in resistant mares [19]. Even though the only IL-6 had significant upregulation in our results for interleukin gene expressions, all these parameters for IL-1β, IL-6, and IL-8 had the highest values at 6 h after insemination with a

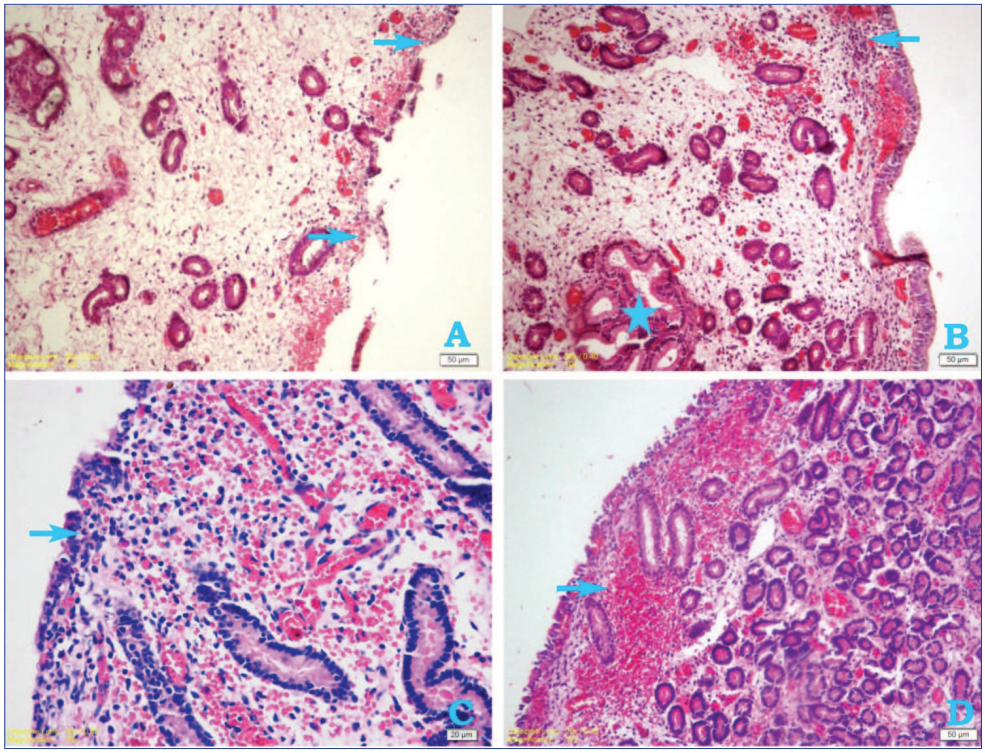

Fig 2. A. Surface epithelial disruption *(arrows),* edema in the mucosa, H&E staining, Bar=50 µm, B. PMNs infiltration in the subepithelial area *(arrow)* and mucosa, nesting uterine glands *(star),* H&E staining, Bar=50 µm, C. Severe PMNs infiltration in the subepithelial area *(arrow)* and mucosa, H&E staining, Bar=20 µm, D. Hemorrhage in the subepithelial area *(arrow),* H&E staining, Bar=50 µm

similar pattern of an up regulation in the first few hours of uterine immune system activation by inflammatory substances as bacterial invasion or sperma.

IL-6, originating from inflammed tissues, is one of the main acute phase proteins. It is suggested that IL-6 initially promotes acute inflammation and PMN recruitment, and subsequently induces PMN apoptosis and phagocytosis which leads to termination of inflammation [9] while IL-8, a crucial pro-inflammatory mediator for chemotaxis [20], is responsible for the continuing migration of PMN's into the uterine lumen [19]. IL-1β is responsible for initiating and down-regulating the pro-inflammatory response [19]. PMN plays role in clearance of inflammatory by-products from uterus by inducing myometrial contractions via stimulating synthesize and secretion of $PGF_{2\alpha}$ from endometrium [5]. Although the absence of statistical significance in double insemination group, mRNA expressions of IL-1β, IL-6, IL-8 were consistent with immunohistochemical findings. As double insemination was performed at 24 h of the study, gene expressions of interleukins which were peaked at 6th h remains higher at 30 h in the present study. There was significance at SED, LP infiltration and F+CD between the groups at 30th h. The higher scores of those parameters in double insemination group represent the inflammatory changes which were moderated by acute phase proteins.

Increased nitric oxide (NO) produced by iNOS serves as an aid in the removal of pathogens but can also be cytotoxic [21-23]. In addition, mares susceptible to PBIE had increased iNOS activity and NO production com pared to resistant mares at 13 h after insemination [11]. Troedsson et al.[24] suggested that contractility deficiencies in susceptible mares between 6 and 19 h after insemination may be associated with increased NO production. However, expression levels of iNOS were not comparable within the single or double insemination groups in our study. According to the endometrial biopsy scores which were determined at beginning of this study, the mares were not susceptible to PBIE. The unchanging NO production after insemination might be explain with the resistance of the mares. The results should be compared with further studies which includes PBIE susceptible mares.

CASP-3 mRNA expression has been detected in placenta and in the uterus of cycling and pregnant cows [25]. The present study is the first report of uterine mRNA expression of CASP-3 in mares after insemination. It is an important enzyme for the first step of apoptosis that can be activated by intrinsic or extrinsic pathways [25]. The rate of apoptosis in the tissue was not taken account in the present study. However, a gradual decrease was observed in CASP-3 expression at each biopsy sampling time after the first insemination in both groups. This decrement might be associated with a potential down-regulation of this protein throughout consecutive sampling times.

In the present study no difference in endometrial gene expression of SAA was found between the two groups. Previous studies have shown an endometrial SAA expression were significantly and rapidly up-regulated in response to inoculation, and *E. coli* endometritis thus provoked a marked and transient inflammatory response [5] but same researcher's another study mRNA expression of SAA was not influenced after *E. coli* infusion. The different expression of SAA between two studies reflected the course of inflammation within the uterus [19].

Neutrophils are the potent producers of cytokines. PMN migration into the uterus peaks about 6 h after experimental introduction of bacteria to the uterine

Table 3. *Histological characteristics of the endometrium immediately before (0 h) and 6, 30 and 54 h after first insemination in single and double single and double insemination cycles*

Histological Inflammatory Parameters	Biopsy Time (hours)	Single Insemination (n=9) Median (Min-Max)	Double Insemination (n=9) Median (Min-Max)	Sig.##
Hyperemia	0	1 (1-2)	2 (1-2)	ns
	6	1 (1-2)	2 (1-2)	ns
	30	1 (1-2)	1 (1-2)	ns
	54	1 (0-2)	1 (1-3)	ns
Sig.#		ns	ns	
Hemorrhage	0	1 (0-3)	1 (0-3)	ns
	6	1 (0-3)	2 (0-3)	ns
	30	1 (0-2)	1 (0-3)	ns
	54	2 (0-3)	2 (0-3)	ns
Sig.#		ns	ns	
Edema	0	2^{ab} (0-3)	2^{a} (1-3)	ns
	6	2^{a} (1-3)	1^{b} (1-3)	ns
	30	1^{b} (0-2)	1^{c} (0-2)	ns
	54	1^{b} (0-2)	2^{ab} (0-3)	ns
Sig.#		*	*	
Surface epithelial disruption	0	1 (0-3)	1^{ac} (0-2)	ns
	6	1 (1-2)	2^{b} (1-2)	ns
	30	1 (0-2)	2^{ab} (0-3)	*
	54	1 (0-2)	1^{c} (0-2)	ns
Sig.#	P	ns	*	
PMN infiltration	0	0^{a} (0-1)	0^{a} (0-1)	ns
	6	2^{b} (0-3)	2^{b} (0-3)	ns
	30	2^{bc} (1-2)	2^{bc} (1-2)	ns
	54	1^{ab} (0-2)	1^{d} (0-1)	ns
Sig.#		*	**	
LP infiltration	0	1 (1-3)	1 (1-2)	ns
	6	1 (0-2)	2 (1-2)	ns
	30	1 (0-2)	2 (1-2)	*
	54	1 (0-2)	1 (1-2)	ns
Sig.#		ns	ns	
Fibrosis and cystic dilation of endometrial glands	0	0 (0-1)	0 (0-3)	ns
	6	0 (0-2)	1 (0-2)	ns
	30	0 (0-3)	1 (0-3)	*
	54	0 (0-3)	2 (0-2)	ns
Sig.#		ns	ns	

Different letters in the column indicate significant differences. ***Sig.#*** *-values for differences within the same insemination cycle.* ***Sig ##*** *-values for differences between single and double insemination cycles. * P<0.05 ** P<0.001* ***ns:*** *P>0.05*

lumen, and normally the response will remain elevated for at least 72 h [26]. The biopsy sampling times (0, 6, 30 and 54 h) of this study was specified with the light of this information. Neutrophil activation was also same between single and double insemination groups. Neutrophils increased after 6 h insemination in both groups and gradually decreased after 54 h insemination with parallel to proinflammatory gene expressions. Other researchers obtained similar results 5-12 h after uterine infusion of bacteria [26,27] and 6 h after natural mating or insemination with chilled or frozen/thawed semen [1]. The increased number of neutrophils after the first insemination was an expected result of this study. However, the number of neutrophils and chemokines like IL-6 and IL-8 which provide chemotaxis for neutrophils did not change after the second insemination. The reason of this result might be the binding between spermatozoa and inflammatory cells after the second insemination in 24 h, as explained by Troedsson [8]. The increase of neutrophils after second

insemination might be detected in uterine lumen rather than uterine tissue.

In conclusion, according to our result, uterine mRNA expressions of IL-1B,-6,-8, iNOS, SAA, COX-2 were not affected by the number of inseminations. In order to establish whether the mares are resistant to PBIE, it is necessary to expose both resistant and susceptible mares to this study and to determine whether gene expression changes are dependent upon the inflammatory status of the uterus. This study provides preliminary evidence to characterize further the changes in the expression of relevant genes in response to live semen. The effect upon gene expression by treatment with other preparations of fresh, chilled or frozen/thawed semen also needs to be investigated in both resistant and susceptible mares.

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REFERENCES

1. Kotilainen T, Hutinen M, Katila T: Sperm induced leukocytosis in the equine uterus. *Theriogenology*, 41, 629-636, 1994. DOI: 10.1016/0093-691X(94)90173-G

2. Watson E: Post-breeding endometritis in the mare. *Anim Reprod Sci*, 60/61, 221-232, 2000. DOI: 10.1016/S0378-4320(00)00110-X

3. Troedsson MH, Liu IK, Thurmond M: Immunoglobulin (IgG and IgA) and complement (C3) concentrations in uterine secretion following an intrauterine challenge of *Streptococcus zooepidemicus* in mares susceptible to versus resistant to chronic uterine infection. *Biol Reprod*, 49, 502–506, 1993. DOI: 10.1016/j.jevs.2012.11.010

4. Troedsson MHT: Uterine clearance and resistance to persistent endometritis in the mare. *Theriogenology*, 52, 461-471, 1999. DOI: 10.1016/S0093-691X(99)00143-0

5. Christoffersen M, Baagoe CD, Jacobsen S, Bojesen AM, Petersen MR, Lehn-Jensen H: Evaluation of systemic acute phase response and endometrial gene expression of serum amyloid A and pro- and anti-inflammatory cytokines in mares with experimentally induced endometritis. *Vet Immunol Immunopathol*, 138, 95-105, 2010. DOI: 10.1016/j.vetimm.2010.07.011

6. Fiala SM, Pimantel CA, Mattos ALG, Gregory RM, Mattos RG: Effect of sperm number and concentration on sperm transport and uterine inflammatory response in the mare. *Theriogenology*, 67, 556-562, 2007. DOI: 10.1016/j.theriogenology.2006.09.005

7. Troedsson MHT, Loset K, Alghamdi AM, Dahms B, Crabo BG: Interactions between equine semen and the endometrium: The inflammatory response to semen. *Anim Reprod Sci*, 68, 273-278, 2001. DOI: 10.1016/S0378-4320(01)00164-6

8. Troedsson MHT: Breeding-induced endometritis in mare. *Vet Clin North Am: Equine Pract*, 22, 705-712, 2006. DOI: 10.1016/j.cveq.2006.07.003

9. Fumuso E, Giguere S, Wade J, Rogan D, Videla-Dorna I, Bowden RA: Endometrial IL-1βeta, IL-6 and TNF-alpha, mRNA expression in mares resistant or susceptible to post-breeding endometritis. Effects of estrous cycle, artificial insemination and immunomodulation. *Vet Immunol Immunopathol*, 96, 31-41, 2003. DOI: 10.1016/S0165-2427(03)00137-5

10. Orsi N, Tribe R: Cytokine networks and the regulation of uterine function in pregnancy and parturition. *J Neuroendocrinol*, 20, 462-469, 2008. DOI: 10.1111/j.1365-2826.2008.01668.x

11. Alghamdi AS, Foster DN, Carlson CS, Troedsson MHT: Nitric oxide levels and nitric oxide synthase expression in uterine samples from mares susceptible and resistant to persistent breeding-induced endometritis. *Am J Reprod Immunol*, 53, 230-237, 2005. DOI: 10.1111/j.1600-0897.2005.00270.x

12. Vane JR, Bakhel YS, Botting RM: Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol*, 38, 97-120, 1998. DOI: 10.1146/annurev.pharmtox.38.1.97

13. FitzGerald GA: COX-2 and beyond: Approaches to prostaglandin inhibition in human disease. *Nat Rev Drug Discov*, 2, 879-890, 2003. DOI: 10.1038/nrd1225

14. Portera AG, Jänicke RU: Emerging roles of caspase-3 in apoptosis. *Cell Death Differ*, 6 (2): 99-104, 1999. DOI: 10.1038/sj.cdd.4400476

15. Kenney RM, Doig PA: Equine endometrial biopsy. **In,** Morrow DA (Ed): Current Therapy in Theriogenology. 723-729, W.B. Saunders Company, Philadelphia, 1986.

16. Bonnett BN, Miller RB, Etherington WG, Martin SW, Johnson WH: Endometrial biopsy in Holstein-Friesian dairy cows I. Technique, histological criteria and results. *Can J Vet Res*, 55, 155-161, 1991.

17. Chapwanya A, Meade KG, Doherty ML, Callanan JJ, Mee JF, O'Farrelly C: Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: Toward an improved understanding of uterine innate immunity. *Theriogenology*, 71 (9): 1396-1407, 2009. DOI: 10.1016/j.theriogenology.2009.01.006

18. Marth CD, Firestone SM, Glenton LY, Brownin, GF, Young ND, Krekeler N: Oestrous cycle-dependent equine uterine immune response to induced infectious endometritis. *Vet Res*, 47, 110, 2016. DOI: 10.1186/s13567-016-0398-x

19. Christoffersen M, Woodward E, Bojesen AM, Jacobsen S, Petersen MR, Troedsson MH, Lehn-Jensen H: Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis. *BMC Vet Res*, 8, 41-54, 2012. DOI: 10.1186/1746-6148-8-41

20. Fumuso E, Aguilar J, Giguere S, David O, Wade J, Rogan D: Interleukin-8 (IL-8) and 10 (IL-10) mRNA transcriptions in the equine endometrium of normal mares and mares susceptible to persistent post-breeding endometritis. *Anim Reprod Sci*, 94, 31-41, 2006. DOI: 10.1016/j.anireprosci.2006.04.006

21. Tripathi P: Nitric oxide and immune response. *Indian J Biochem Biophys*, 44, 310-319, 2007. DOI: 10.1016/j.it.2015.01.003

22. MacMicking J, Xie QW, Nathan C: Nitric oxide and macrophage function. *Annu Rev Immunol*, 15, 323-350, 1997. DOI: 10.1146/annurev.immunol.15.1.323

23. Kurar E, Atlı MO, Guzeloglu A, Semacan A: POMC, iNOS, PGES, IL-4, IL-5 and IL-10 gene expression in peripheral blood mononuclear cells of cyclic and pregnant mares. *Kafkas Univ Vet Fak Derg*, 17 (2): 319-323, 2011. DOI: 10.9775/kvfd.2010.3644

24. Troedsson MHT, Liu IK, Ing M, Pascoe J, Thurmond M: Multiple site electromyography recordings of uterine activity following an intrauterine bacterial challenge in mares susceptible and resistant to chronic uterine infection. *J Reprod Fertil*, 99, 307-313, 1993. DOI: 10.1530/jrf.0.0990307

25. Moares CN, Maia L, Lima PF, Dias MC, Raposo-Ferreira, TMM, Sudano MJ, Junior JB, Oba E: Temporal analysis of prostaglandin F2α receptor, caspase 3, and cyclooxygenase 2 messenger RNA expression and prostaglandin F2α receptor and cyclooxygenase 2 protein expression in endometrial tissue from multiparous Nelore *(Bos taurus indicus)* cows treated with cloprostenol sodium during puerperium. *Theriogenology*, 83, 276-284, 2015. DOI: 10.1016/j.theriogenology.2014.09.022

26. Troedsson MH, Liu IK, Thurmond M: Function of uterine and blood-derived polymorphonuclear neutrophils in mares susceptible and resistant to chronic uterine infection: Phagocytosis and chemotaxis. *Biol Reprod*, 49, 507-514, 1993. DOI: 10.1095/biolreprod49.3.507

27. Katila T, Lock T, Hoffman W, Smith A: Lysozyme, alkaline phosphatase and neutrophils in uterne secretions of mares with different resistance to endometritis. *Theriogenology*, 33, 723-732, 1990. DOI: 10.1016/0093-691X(90)90549-9